American Journal of Pathology, Vol. 143, No. 3, September 1993
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Microtubule Antagonists Activate Programmed Cell Death (Apoptosis) in Cultured Rat Hepatocytes

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We investigated the mechanism of lethal injury following the disruption of microtubules in cultured hepatocytes treated with vinblastine (VBL) or colchicine (COL). These agents kill hepatocytes by a process readily distinguished from two well-known pathways that lead to a loss of viability, namely, oxidative stress and inhibition of mitochondrial electron transport. Cell killing with VBL and COL was accompanied by fragmentation of DNA. Both the loss of viability and the fragmentation of DNA were prevented by the inhibition of protein synthesis within 6 hours following exposure to VBL or COL. Cell death and the fragmentation of DNA were also prevented when Ca^{2+} was removed from the culture medium. By contrast, the inhibition of protein kinase C prevented cell killing by VBL or COL, but did not alter the extent of DNA fragmentation. The requirements here for protein synthesis, extracellular Ca^{2+}, and protein kinase C activity define a model of apoptosis, or programmed cell death, that seems to involve mechanisms that can be dissociated from the fragmentation of DNA. (Am J Pathol 1993, 143: 918–925)

Programmed cell death is an active process exemplified by the elimination of tissue that occurs during embryonic development.[1,2] The process is accompanied by an internucleosomal fragmentation of DNA that is widely held to distinguish programmed cell death from toxic injury and necrosis.[3–5] Recently, the concept of programmed cell death has been broadened to include a variety of injuries characterized by DNA fragmentation (DNA-F) and referred to as apoptosis. These include the spontaneous or programmed death of tumor cells[6,7] and the killing of various cell types by hormones,[8,9] ionizing radiation,[10] toxic chemicals,[11,12] and various cytostatic drugs,[13–16] including cisplatin, etoposide, and methotrexate.

In most cases where cell death is attributed to apoptosis, the molecular mechanisms remain poorly understood. In large part, this may be a consequence of the fact that mere demonstration of DNA-F has increasingly been used to define any toxic injury as an example of apoptosis.[17,18] In turn, there is a general tendency to characterize death by apoptosis as an inevitable consequence of a primary DNA-F. In most cases, however, the mechanisms leading to DNA-F and their relationship to the loss of cell viability remain to be defined. Furthermore, it is not clear that many of the examples of apoptosis in the literature represent the earlier concept of programmed cell death as an active process.

The present report describes the activation of a mechanism of programmed cell death in cultured rat hepatocytes intoxicated with microtubule antagonists. In this model, cell killing requires protein synthesis and is accompanied by, but is not the inevitable consequence of, DNA-F. Thus, the data define an example of apoptosis that is the consequence of an active process that would seem to result from mechanisms that can be dissociated from DNA-F.

Materials and Methods

Male Sprague-Dawley rats (150 to 200 g, Charles River Breeding Laboratories, Inc., Wilmington, MA) were fed *ad libitum* and then fasted overnight before use. Isolated hepatocytes were prepared by the collagenase (Sigma Chemical Co., St. Louis, MO) perfusion method of Seglen.[19] Yields of 2 to 4

Supported by NIH grants DK38305 and AA07186.

Accepted for publication May 4, 1993.

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$\times 10^8$ cells/liver with 85 to 90% viability by trypan blue exclusion were routinely obtained. Hepatocytes were plated in 25-cm^2 flasks (Corning Glass Works, Corning, NY) at a density of 1.33×10^6 cells/flask in complete Williams E medium (GIBCO Laboratories, Grand Island, NY) containing 10 IU/ml penicillin, 10 μg/ml streptomycin, 50 μg/ml gentamycin, 0.02 U/ml insulin, and 10% heat-inactivated (55 C for 15 minutes) fetal bovine serum (JRH Biosciences, Kansas City, MO). After incubation for 2 hours at 37 C in an atmosphere of 5% CO_2-95% air, the cultures were washed twice with prewarmed 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, Sigma) buffer (140 mmol/L NaCl, 6.7 mmol/L KCl, 1.2 mmol/L $CaCl_2$ and 2.4 mmol/L HEPES, pH 7.4) to remove unattached or dead cells. Complete Williams E was replaced, and flasks were further incubated for 1 hour until the pH of the culture medium was stable. Additions were made as indicated in the text. For experiments performed in the absence of extracellular Ca^{2+}, the cultures were washed and incubated in serum-free, Ca^{2+}-free Williams E medium with additions as noted. All experiments were performed on at least three separate cultures and were repeated with at least three separate preparations of hepatocytes on different days. In every case, the data shown are from one experiment and are always representative of the three. The variability in the viability of control cells after 24 hours reflects the inherent variation between preparations of isolated hepatocytes.

The viability of the cultured cells was determined by the release of lactate dehydrogenase activity as described previously.[20] DNA-F in hepatocyte cultures was measured following lysis (0.3% v/v Triton X-100, 3 mmol/L Tris, 12 mmol/L ethylenediaminetetraacetic acid, pH 8.0) and centrifugation (27,000*g*, 20 minutes) to separate intact chromatin from DNA fragments.[3,21] Pellets containing nonfragmented DNA were resuspended in 1 N perchloric acid, whereas the supernatants containing fragmented DNA were further centrifuged (27,000*g*, 15 minutes) following addition of perchloric acid. The second pellet was also resuspended in 1 N perchloric acid. Both pellets were heated (70 C, 20 minutes), centrifuged (3,000*g*, 5 minutes), and assayed colorimetrically for DNA content using the diphenylamine reagent.[22] DNA-F was expressed as the percentage of total DNA appearing in the original supernatant.

DNA-F was also evaluated qualitatively by agarose gel electrophoresis. Lysates obtained as described above were treated with 0.5 mol/L NaCl in 50% isopropanol overnight at −20 C.[23] Following centrifugation (27,000*g*, 15 minutes), the pellet was air-dried, resuspended in sodium dodecyl sulfate buffer (0.1 mol/L ethylenediaminetetraacetic acid, 20 mmol/L NaCl, 0.5% sodium dodecyl sulfate, pH 8.0), and treated with 100 μg/ml proteinase K overnight at 50 C. DNA was extracted with phenol and phenol/chloroform (1:1) and precipitated with ethanol/sodium acetate overnight at −20 C.[24] DNA samples were separated by electrophoresis on 0.8% agarose gel containing 0.4 μg/ml ethidium bromide. A 1-kb DNA Ladder provided standards for molecular size.

HEPES, collagenase, cycloheximide (CHX), vinblastine (VBL), colchicine (COL), potassium cyanide (KCN), *tert*-butyl hydroperoxide (TBHP), 1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride (H-7), staurosporine (STS), ethidium bromide, and protease K were obtained from Sigma. Agarose was from Fisher Chemical Co. (Fair Lawn, NJ), phenol from Boehringer Mannheim Corporation (Indianapolis, IN), and 1-kb DNA Ladder from GIBCO Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, MD).

Results

The Killing of Cultured Hepatocytes by VBL and COL

Figure 1 illustrates the time course of the cell killing (open circles) and fragmentation of DNA (closed circles) that occurred with 0.5 μg/ml of VBL and 0.5 μmol/L COL. Significant loss of viability required at least 12 hours of exposure to either agent. After this time, cell death increased steadily to reach between 40 and 60% of the cells by 24 hours. Evidence of DNA-F preceded the loss of viability with both VBL and COL. Within 12 hours of exposure to either agent, there was significant DNA-F with no loss of viability with VBL and only minimal cell killing with COL. DNA-F increased between 12 and 24 hours with VBL and COL in parallel with the increased cell killing. This close correlation between cell killing and DNA-F was observed over the dose range of 0.1 μg to 1 μg/ml of VBL (0.12 mmol/L to 1.23 mmol/L) and 0.1 μmol/L to 1 μmol/L COL (data not shown).

By contrast, Figure 1 illustrates that the cell killing by 1 mmol/L KCN occurs over a faster time and is not preceded by DNA-F. After 6 hours, 80% of the cell had been killed without evidence of DNA-F. By 12 hours, 95% of the cells were dead with minimal DNA-F. The DNA-F that follows the cell killing by

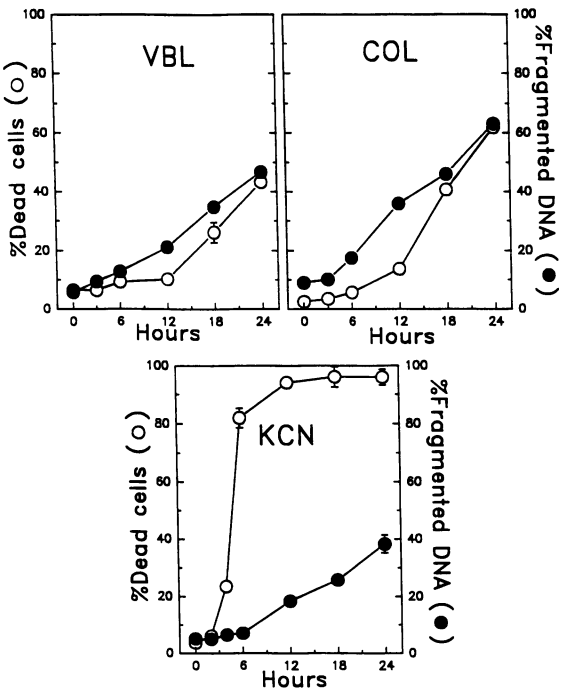

Figure 1. *Time course of cell killing and DNA-F in cultured hepatocytes treated with 0.5 μg/ml of VBL, 0.5 μmol/L COL, or 1 mmol/L cyanide. The results represent the mean ± SD of three separate cultures. Where the error bars are not apparent, the SD was less than the size of the data point. One experiment representative of three is illustrated.*

KCN most likely reflects the autolytic degradation that inevitably accompanies the death of cells and does not imply that the cells have died by apoptosis. Similarly, the killing of virtually all of the hepatocytes with 0.5 to 1.0 mmol/L TBHP was not accompanied by any evidence of DNA-F (data not shown).

DNA-F by Agarose Gel Electrophoresis

Figure 2 documents by agarose gel electrophoresis the fragmentation of DNA in hepatocytes intoxicated with VBL and COL. The characteristic ladder pattern of internucleosomal DNA cleavage consistent with activation of an endogenous endonuclease is readily evident. Using the 1-kb DNA Ladder as a marker for size, these fragmentation patterns most likely represent 200-bp multiples of DNA. DNA from untreated cells or hepatocytes intoxicated with KCN or TBHP remained relatively unfragmented.

Protection by Inhibition of Protein Synthesis

Table 1 documents the protection afforded by an inhibition of protein synthesis with CHX. With VBL, the simultaneous addition of 1 μmol/L CHX reduced by

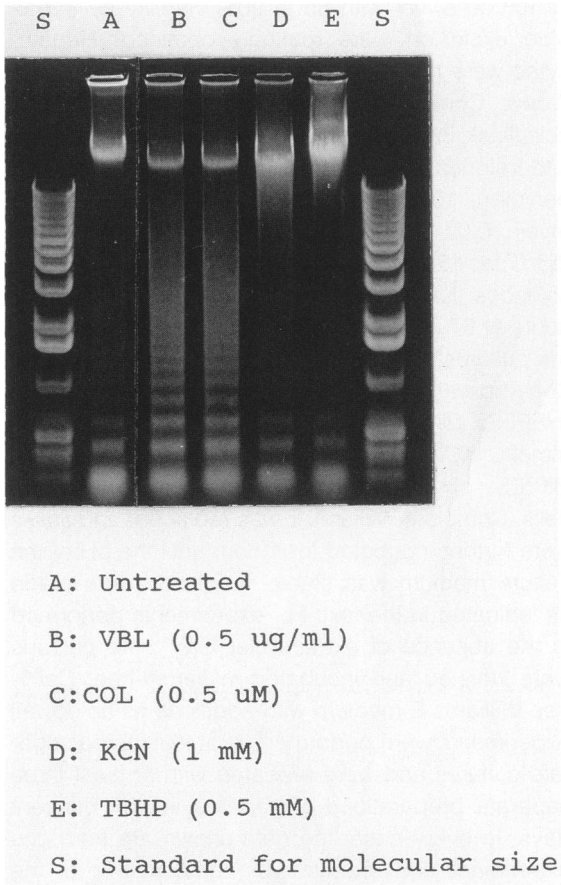

Figure 2. *Agarose gel electrophoresis of DNA of hepatocytes intoxicated with VBL or COL.*

Table 1. *Cycloheximide Prevents the Loss of Viability and Fragmentation of DNA in Hepatocyte Cultures Treated with VBL or COL*

Treatment	Cell death (% ± SD)	DNA fragmentation (% ± SD)
None	12.8 ± 0.8	19.1 ± 0.6
CHX (1 μmol/L)	12.2 ± 0.4	16.7 ± 0.4
VBL (0.5 μg/ml)	44.8 ± 0.8	40.9 ± 0.6
VBL plus CHX	22.5 ± 0.4	23.1 ± 2.2
COL (0.5 μmol/L)	35.5 ± 1.4	41.7 ± 0.5
COL plus CHX	15.7 ± 0.8	22.7 ± 0.6

Cell killing was determined after a 22-hour exposure to VBL or COL. The results represent the mean ± SD on three separate cultures. One experiment representative of three is illustrated.

at least two-thirds the extent of both the cell killing and the fragmentation of DNA. Similarly, in cultures treated with COL, CHX afforded virtually complete protection against both the loss of viability and DNA-F. CHX had no effect on the cell killing by either KCN or TBHP (data not shown). The dose of CHX used here inhibited the incorporation of [^{3}H]-leucine into total protein by 85% (data not shown).

Table 2 shows the effect of the time of addition of CHX on the loss of viability and DNA-F in cultures

Table 2. *The Time of Addition of Cycloheximide Influences the Loss of Viability and Fragmentation of DNA in Cultured Hepatocytes Treated with VBL or COL*

Treatment	Cell death (% ± SD)	DNA fragmentation (% ± SD)
None	9.6 ± 0.6	18.5 ± 0.9
CHX (1 μmol/L)	11.4 ± 2.0	18.9 ± 1.6
VBL (0.5 μg/ml)	45.5 ± 1.3	47.4 ± 0.8
VBL plus CHX (Time 0)	24.0 ± 1.1	28.8 ± 0.5
VBL plus CHX (3 hr)	23.4 ± 2.5	32.9 ± 1.4
VBL plus CHX (6 hr)	13.1 ± 1.5	23.4 ± 0.2
VBL plus CHX (8 hr)	21.1 ± 0.7	25.9 ± 0.4
VBL plus CHX (10 hr)	31.6 ± 1.0	31.2 ± 2.2
VBL plus CHX (12 hr)	38.4 ± 0.9	35.8 ± 1.8
COL (0.5 μmol/L)	53.1 ± 0.8	50.6 ± 2.1
COL plus CHX (Time 0)	22.8 ± 1.1	23.7 ± 1.6
COL plus CHX (6 hr)	21.0 ± 0.9	23.7 ± 2.2

CHX was added at various intervals following the addition of VBL or COL. Cell death and DNA fragmentation were measured 24 hours after the addition of VBL or COL. The results represent the mean ± SD on three separate cultures. One experiment representative of three is illustrated.

treated with VBL or COL. The protective effect of CHX against both the cell killing and DNA-F was maximal when the drug was added 6 hours after treatment with VBL. The delay in the addition of CHX for 6 hours was somewhat more effective in protecting the cells than adding it at time 0. This may simply relate to the fact that in the absence of inhibition of protein synthesis for 6 hours, the cells are better able to withstand the double shock of their isolation and subsequent exposure to VBL. Prevention of cell killing and DNA-F decreased when CHX was added more than 6 to 8 hours after the VBL.

Addition of CHX could also be delayed until 6 hours after treatment of the hepatocytes with COL. There was no difference in the extent of the reduction in either cell killing or DNA-F when CHX was added together with or 6 hours after COL.

Protection by Removal of Extracellular Calcium

The culture medium used in the above experiments (Williams E) contains 2.8 mmol/L $CaCl_2$. Table 3 addresses the role of this extracellular Ca^{2+} in the killing of cultured hepatocytes by VBL or COL. By treating the hepatocytes with these agents in a culture medium from which calcium ions had been removed (<20 μmol/L Ca^{2+}), the extent of cell killing was reduced by as much as 75%. Removal of extracellular calcium ions similarly reduced the extent of DNA-F.

Table 4 explores the effect of the time of removal of extracellular calcium on the loss of viability and the fragmentation of DNA in cultures treated with VBL or COL. Removal of extracellular calcium ions

Table 3. *Removal of Extracellular Calcium Prevents the Loss of Viability and Fragmentation of DNA in Hepatocyte Cultures Treated with VBL or COL*

Treatment	Cell death (% ± SD)	DNA fragmentation (% ± SD)
Control	10.4 ± 0.3	11.8 ± 0.1
Control minus Ca^{2+}	4.5 ± 0.3	7.4 ± 0.3
VBL (0.5 μg/ml)	50.0 ± 1.6	46.1 ± 0.7
VBL minus Ca^{2+}	22.0 ± 1.8	23.8 ± 0.3
COL (0.5 μmol/L)	51.0 ± 1.2	46.2 ± 0.5
COL minus Ca^{2+}	26.5 ± 0.7	27.0 ± 0.4

Cell killing was determined after a 24-hour exposure to VBL or COL. The results represent the mean ± SD on three separate cultures. One experiment representative of three is illustrated.

after 6 hours of exposure to either VBL or COL afforded a greater protection than the removal of calcium during the first 6 hours of exposure to either agent. The effect of the time of removal of extracellular calcium on the extent of DNA-F was less pronounced.

Protection by Inhibitors of Protein Kinase C (PKC)

PKC activity has been implicated in the mechanism of cell killing in several models held to represent programmed cell death.[25] Table 5 illustrates the ability of two different PKC inhibitors, H-7 and STS, to prevent the killing of cultured hepatocytes by VBL or COL. When added together with VBL, both H-7 and STS decreased the loss of viability by as much as 60 to 70%. Under the same conditions, H-7 and STS did not alter the extent of DNA-F. A similar dissociation of cell killing from DNA-F was obtained when either H-7 or STS was added 6 hours after

Table 4. *The Time of Removal of Extracellular Calcium Influences the Loss of Viability and Fragmentation of DNA in Cultured Hepatocytes Treated with VBL or COL*

Treatment	Cell death (% ± SD)	DNA fragmentation (% ± SD)
Control plus Ca^{2+}	19.6 ± 0.7	23.7 ± 0.7
Control minus Ca^{2+}	9.2 ± 0.4	13.4 ± 0.5
Control minus Ca^{2+} (6–24 hr)	6.7 ± 0.4	11.1 ± 0.2
Control minus Ca^{2+} (0–6 hr)	14.3 ± 0.4	18.5 ± 0.7
VBL (0.5 μg/ml)	61.8 ± 0.4	50.0 ± 1.1
VBL minus Ca^{2+}	36.9 ± 3.3	35.9 ± 1.0
VBL minus Ca^{2+} (6–24 hr)	29.9 ± 1.0	41.8 ± 1.4
VBL minus Ca^{2+} (0–6 hr)	46.5 ± 1.0	47.9 ± 0.6
COL (0.5 μmol/L)	55.4 ± 0.4	51.0 ± 2.0
COL minus Ca^{2+}	30.6 ± 0.9	37.2 ± 1.5
COL minus Ca^{2+} (6–24 hr)	27.0 ± 0.9	38.1 ± 1.2
COL minus Ca^{2+} (0–6 hr)	40.2 ± 0.8	45.2 ± 1.7

Cell killing was determined after a 24-hour exposure to VBL or COL. The results represent the mean ± SD on three separate cultures. One experiment representative of three is illustrated.

Table 5. *Inhibitors of PKC Prevent the Loss of Viability but not the Fragmentation of DNA in Hepatocyte Cultures Treated with VBL or COL*

Treatment	Cell death (% ± SD)	DNA fragmentation (% ± SD)
None	7.9 ± 0.4	12.6 ± 0.6
H-7 (100 μmol/L)	11.9 ± 0.4	20.0 ± 0.1
STS (1 μmol/L)	8.9 ± 0.4	18.1 ± 0.6
VBL (0.5 μg/ml)	37.5 ± 1.2	40.6 ± 1.7
VBL plus H-7 (time 0)	15.1 ± 1.0	38.7 ± 1.8
VBL plus H-7 (6–24 hr)	18.3 ± 1.2	37.2 ± 0.5
VBL plus STS (time 0)	15.4 ± 0.6	46.3 ± 1.8
VBL plus STS (6–24 hr)	17.9 ± 1.2	51.5 ± 3.0
COL (0.5 μmol/L)	34.7 ± 0.5	34.6 ± 1.2
COL plus H-7 (time 0)	16.7 ± 0.9	29.2 ± 2.2
COL plus H-7 (6–24 hr)	18.0 ± 0.5	22.1 ± 0.8
COL plus STS (time 0)	16.9 ± 0.9	39.7 ± 0.5
COL plus STS (6–24 hr)	15.0 ± 0.5	43.4 ± 2.2

Cell killing was determined after a 24-hour exposure to VBL or COL. The results represent the mean ± SD on three separate cultures. One experiment representative of three is illustrated.

treatment with VBL. H-7 and STS had no effect on the killing of cultured hepatocytes by either KCN or TBHP (data not shown).

The two PKC inhibitors also prevented the cell killing by COL. The effect on DNA-F was less pronounced. The dissociation of cell killing from DNA-F was particularly striking with STS. When added together with COL or 6 hours after COL, STS reduced the loss of viability by as much as 60 to 70% without any reduction in the extent of DNA-F.

Discussion

The data presented above document that VBL and COL kill cultured hepatocytes by a mechanism that can readily be distinguished from two well-known pathways that lead to a loss of viability, namely, oxidative stress[26] and inhibition of mitochondrial electron transport.[27] DNA-F preceded or accompanied the killing of cultured hepatocytes by VBL and COL (Figure 1). By contrast, with TBHP (oxidative stress) and KCN (inhibition of electron transport), DNA-F was not evident until the cells had died. Cell killing by VBL and COL depended on protein synthesis and was prevented by its inhibition with CHX (Table 1). By contrast, CHX had no effect on the toxicity of TBHP or KCN. Removal of calcium ions from the culture medium similarly prevented the cell killing by VBL and COL, but was without effect on that by TBHP or KCN. Finally, inhibition of PKC with either H-7 or STS reduced the toxicity of VBL and COL. The cell killing by TBHP or KCN was unaffected by these same agents.

The characteristics of the killing of cultured hepatocytes by VBL and COL are consistent with activation-induced or programmed cell death, responses commonly included under the rubric of apoptosis. We suggest that the disruption of the integrity of microtubules by VBL or COL is recognized by the hepatocytes. The cells respond with the

synthesis of a protein(s) that, in turn, triggers the killing of the cells. The mechanism of lethal injury requires the presence of extracellular calcium ions and the participation of PKC.

The function of liver cells includes, to a large extent, the release to and extraction from the blood of numerous metabolic products. This function requires the integrity of the microtubular apparatus. Thus, the disruption of microtubules would render the hepatocyte functionally useless, but not necessarily lethally injured. That such incompetent cells can be eliminated by activation of a cell killing program is clearly advantageous, because regeneration should readily restore functionally competent liver cells.

The data presented here document three requirements for the programmed killing of cultured hepatocytes by VBL or COL, namely, protein synthesis, extracellular calcium ions, and PKC activity. The nature of the protein(s) synthesized beyond 6 hours remains to be defined. However, because DNA-F could be dissociated from the loss of viability, it is unlikely that CHX is inhibiting the synthesis of a protein required for the internucleosomal disruption of DNA, presumably by activation of an endonuclease.

The requirement for extracellular calcium most likely relates to an increased flux of these ions across the plasma membrane of injured hepatocytes. It is not unreasonable to suggest that the requirement for protein synthesis may be related to such an increased permeability to extracellular calcium ions. More specifically, there is precedent for the hypothesis that cell killing depends on the production of a protein that alters the permeability of the plasma membrane. Such a situation occurs with the killing of cells by virus, either directly or indirectly with the participation of the immune system.

A rise in the cytosolic concentration of Ca^{2+} is required for the activation of PKC under various conditions,[25] and there are many reports to suggest that PKC may play an important role in programmed cell death.[28,29] Proposed mechanisms by which PKC may modulate, enhance, or trigger programmed cell death include: a) receptor-mediated mobilization and maintenance of high levels of $[Ca^{2+}]_i$, b) phosphorylation of proteins involved in metabolism, gene expression, or the structural integrity of the cell, and c) the synthesis of cytotoxic lymphokines (e.g., tumor necrosis factor) or other proteins.[25] It has been suggested that sustained translocation and activation of PKC with subsequent protein phosphorylation might be a critical event leading to the uncontrolled influx of extracellular Ca^{2+} and subsequent cell death in other models of apoptosis.[25]

Whereas inhibitors of PKC afforded substantial protection from the cell killing by VBL or COL, they were less effective in preventing the fragmentation of DNA. By contrast, removal of extracellular calcium reduced the cell killing and DNA-F in parallel. Taken together, these data suggest that a rise of cytosolic Ca^{2+} may activate PKC, an event that is required for lethal injury (Figure 3). At the same time, a rise in the intracellular content of calcium activates an endonuclease with the resultant fragmentation of DNA. However, this second effect of the influx of Ca^{2+} does not depend on PKC activation and is not necessarily related to the lethal injury.

This conclusion is consistent with previous reports that the key morphological features of apoptosis may occur in the absence of internucleosomal DNA-F.[30–32] Moreover, endonuclease is constitutively present in intact, nonapoptotic nuclei and may be activated by mechanisms unrelated to apoptosis.[33] Therefore, endonuclease activation should not

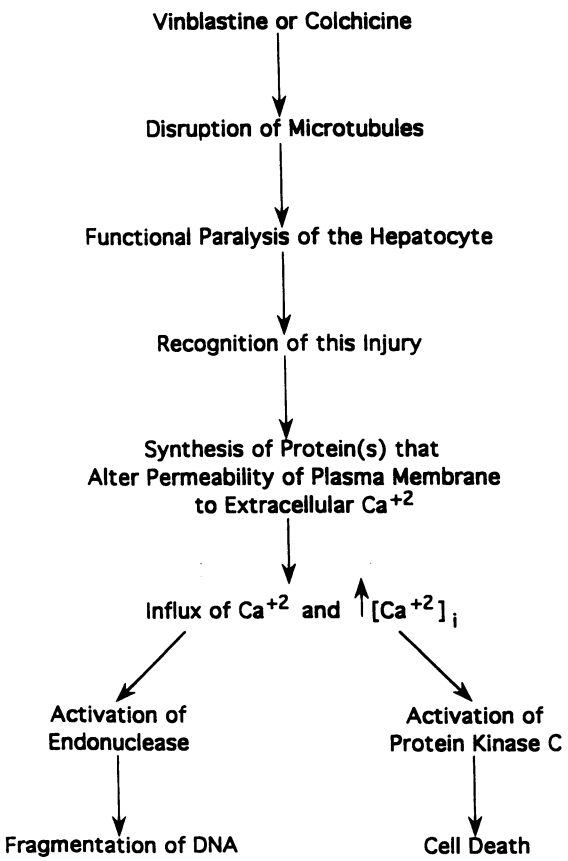

Figure 3. *Proposed mechanism of the cell killing by VBL or COL.*

be considered a general marker or sine qua non for apoptosis,[34] and the assessment of programmed cell death by biochemical assays alone should be interpreted with caution. Our data provide further support for the hypothesis that there are several mechanisms responsible for the initiation of apoptosis that may exhibit both cellular and organ specificity.

It needs to be emphasized that we have defined a distinct pathway to lethal injury that may or may not correspond to what others have called programmed cell death or, more generally, apoptosis. Whether this pathway in the cultured hepatocytes includes the classic morphological changes that some have used to define apoptosis is clearly of interest but, importantly, is not relevant to the interpretation of the data presented. In fact, a careful morphological analysis of the cells is currently underway in our laboratory, the results of which will be interpreted in the light of the results of the present study (and not vice versa).

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